FORM PTO-1390(Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **15317**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): **Unassigned** 10/069914

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/AU00/01026	**30 August 2000 (30.08.00)**	**30 August 1999 (30.08.99)**

TITLE OF INVENTION
TREATMENT OF PANCREATIC DISEASE

APPLICANT(S) FOR DO/EO/US
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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3))
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Courtesy copy of International Application including amendments made pursuant to PCT Article 34
Twenty-one (21) sheets of drawings

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/069914 Unassigned	PCT/AU00/01026	15317

24. The following fees are submitted:	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
☒ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$1,040.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$130.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	22 - 20 =	2	x $18.00	**$36.00**	
Independent claims	4 - 3 =	1	x $84.00	**$84.00**	
Multiple Dependent Claims **(check if applicable)**			☒	**$280.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,570.00**	
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$0.00**	
SUBTOTAL =				**$1,570.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$1,570.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)) The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$1,570.00**	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$1,570.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **19-1013/SSMP** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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TREATMENT OF PANCREATIC DISEASE

Field of Invention

The present invention relates to treatment of pancreatic diseases of humans and animals. This invention has particular but not exclusive application for treatment of acinar cell carcinoma, mixed cell (including acinar cell) pancreatic carcinoma, acute and chronic pancreatitis.

Prior Art

The pancreas is a secretory gland comprising approximately 80% of acinar cells, 1% to 2% of islet cells in clusters, and 10% to 15% of single layered cuboidal ductal cells interlaced with blood vessels, lymphatics, nerves, and collagenous stroma (Evans et al. Cancer of the Pancreas. In Cancer: Principles and Practice of Oncology, 5th edition, DeVita et al. (Eds), Lippincott-Raven, New York).

Despite the large population of acinar cells, acinar cell carcinoma only accounts for 1% - 3% of pancreatic carcinomas. In addition only 5% - 10% of pancreatic carcinomas comprise mixed cell populations including acinar cells (Nonomura et al., 1992 Ultrastructural Pathology 16:317-329; Cubilla and Fitzgerald 1975 Cancer Research 35:2234-2248). Pancreatic mixed cell carcinomas and acinar cell carcinomas have been reported to be aggressive diseases with a high fatality rate (Klimstra et al., 1992, Am. J. Surg. Pathol. 16(9):815-837; Adis Editors. 1997, The Oncology Review 2-4). Surgical resection is the recommended treatment for pancreatic carcinomas. However even with reductions in operative mortality

little from its initial description in 1935 with the current 5 year survival rate being between 2% and 5% (Adis Editors. 1997, The Oncology Review 2-4).

Another disease of the pancreas is acute pancreatitis which appears to have variable severity. Acute pancreatitis appears to arise when the pancreatic duct is obstructed by a gallstone or tumour, or when toxins to the pancreas such as ethanol are ingested. Enzyme production continues causing digestion of the pancreas with often fatal results. Recurrent bouts of acute pancreatitis (or chronic pancreatitis) often result in scarring and deformation of the ductal system thereby causing localised obstruction and thus perpetuating pain, disability and digestive deficiency.

The events which regulate the severity of acute pancreatitis are unknown. Several studies, however, have shown that mild pancreatitis was found to be associated with extensive apoptotic acinar cell death while severe pancreatitis was noted to involve extensive acinar cell necrosis but very little acinar cell apoptosis (Kaiser et al. 1995, Am. J. Physiol. 269:C1295-C1304: Gukovskaya et al. 1996 Gastroenterology 110:875-884).

Administration of cyanohydroxybutene (CHB) to a subject appears to affect *inter alia* the pancreas. CHB is a glycosinolate breakdown product found in cruciferous vegetables, raw canola, and many stock feeds. It was observed that CHB administered at a daily dose of 200 mg/kg to rats by gavage for four days caused acinar cell apoptosis, inflammation, and exocrine pancreatic atrophy (Wallig et al. 1988, Fd Chem Toxic 26:137-147).

Histological and ultrastructural evaluations have been conducted on rats at different time periods after administration by gavage of 200 mg CHB/kg body weight in corn oil. These investigations revealed that as early as 4 hours after CHB administration, the pancreas exhibited abnormal pathology including mild to

moderate supranuclear vacuolation of acinar cells. After 24 hours of CHB administration, the rats exhibited acinar cell apoptosis with cytoplasmic basophilia, lack of zymogen, diffuse vacuolation, clumping of chromatin, and nuclear pyknosis or karyorrhexis (Wallig and Jeffery 1990, Fund. Appl. Toxicol. 14:144-159).

Synthetic CHB being racemic mixture of the R- and S- enantiomers administered by gavage in olive oil at doses of 25-200 mg/kg body weight causes similar effects in the pancreas of rats as naturally occurring CHB. It was observed that pancreatic edema and acinar cell vacuolation and depletion of zymogen granules occurred within hours of administration (Maher et al. 1991, Pancreas 6:168-175).

A single dose of 50 mg CHB/kg was administered intravenously to rats and found to form apoptotic bodies in the pancreas whereas a single dose of 100mg CHB/kg was found to cause severe pancretotoxicity with necrosis (Wallig et al. 1992 Fundamental Applied Toxicology 19:598-606).

In a further study the relationship between acinar cell apoptosis and the severity of pancreatitis was investigated by administering a single intravenous dose of CHB (70mg/kg) in corn oil to mice and inducing pancreatitis at varying times after CHB administration. They found that the severity of pancreatitis is reduced when the disease is induced during the period in which apoptosis is most extensive. However induction of pancreatitis either before or after the peak period of apoptosis results in pancreatic injury which is similar to that noted in animals not exposed to CHB (Bhatia et al.1998, Biochem. Biophys. Res. Commun. 246:476-483). It appears that the acinar cells may regenerate after treatment with CHB.

Summary of the Invention

The present invention arises from the surprising discovery that subcutaneous injection of CHB produces an unusual and unsuspected result of acinar cell apoptosis of normal acinar cells and acinar cell carcinoma. It was found that subcutaneous injection of CHB at an appropriate sub-lethal dosage caused apoptosis of the substantially entire population of acinar cells. The pancreatic lesion was unusual in that there was observed a marked early edema with limited inflammatory infiltration, rapid synchronous onset of acinar cell apoptosis and advanced atrophy with only a severely limited regenerative response. The application of this discovery to treat acinar cell carcinoma has led to the development of the current invention.

The present invention in one aspect broadly resides in a method of providing selective, substantially total, non-regenerative apoptosis of pancreatic acinar cells comprising a single-dose, subcutaneous or intra-arterial administration of a composition of cyanohydroxybutene and a pharmacologically acceptable aqueous carrier

An amount within the therapeutic window is the amount below a lethal dose that has the desired therapeutic effect. Preferably, the therapeutic window is selected whereby CHB serum levels are maintained at a level and for a period sufficient to cause apoptosis of substantially the whole acinar cell population, whilst remaining below the threshold serum levels that cause undesirable levels of liver damage.

The patient may be selected whereby the acinar cells being treated include acinar carcinoma cells. The acinar carcinoma cells may be presenting as localized to the pancreas or as metastases.

The administration is preferably subcutaneously, intramuscularly or intra-arterial catheter direct to the pancreas. These modes of administration appear to

provide CHB in the blood at a more even rate to form a concentration plateau and substantially reduce the height of the concentration peak of CHB. Oral and intravenous administration appears to produce concentration CHB peaks. Oral and intravenous administration of high doses of CHB causes substantial damage to the liver.

In a further aspect, this invention resides broadly in a method for treating pancreatic disease including administering to a patient a therapeutically effective amount of cyanohydroxybutene to cause substantially non-regenerative apoptosis of acinar cells in the patient.

The invention in another aspect resides broadly in a method of treating pancreatic carcinoma having acinar cells including

preparing a cyanohydroxybutene (CHB) formulation; and

administering one or more sub-lethal doses of the CHB formulation to a subject with acinar cell carcinoma, wherein the treatment causes substantially non-regenerative apoptosis of malignant acinar cells in a patient.

The CHB formulation is preferably a CHB solution wherein CHB is substantially dissolved in water. The CHB may be a natural or synthetically derived CHB.

The CHB dose is preferably calculated within a range of 5-300mg CHB/kg of body weight. Preferably the CHB dose is within the range of 125-160 mg CHB/kg of body weight. More preferably the CHB dose is approximately 150 mg CHB/ kg of body weight. The dosage may vary between subjects. Subjects include animals and people of different sizes and weight.

Preferably only one dose is administered. However a second or subsequent dose may be administered but preferably after a period of time such as a week or when the glutathione levels have returned to approximately normal levels.

Administration is preferably by means where the CHB is absorbed comparatively slowly and thus substantially avoids the development of CHB serum concentration peaks that cause undesirable levels of damage to the liver. Preferably the CHB dose is administered by subcutaneous injection, intramuscularly or intra-arterial catheter direct to the pancreas. In an alternative form the CHB dose is administered so that it is delivered directly to the acinar cells. In this form the CHB molecule may be conjugated to a ligand molecule which is able to bind to an acinar cell surface receptor thereby delivering CHB to the acinar cell.

Acinar cell carcinoma includes carcinomas of only acinar cells or of mixed cell populations with a portion being acinar cells.

The above method of treating acinar cells may be applied to the treatment of acute and chronic pancreatitis. Thus in another aspect the present invention broadly resides in a method for treating acute or chronic pancreatitis including

preparing a cyanohydroxybutene (CHB) formulation; and

administering one or more sub-lethal doses of the CHB formulation to a subject with acute or chronic pancreatitis wherein the treatment includes an amount of CHB that causes apoptosis of substantially all acinar cells and substantially no regeneration of acinar cells.

The description of the features of the method for treating acinar cell carcinoma apply also to the above method where applicable.

Brief description of the Drawings

In order that the invention may be more readily understood reference will now be made to the accompanying drawings which illustrate the experimental results and a preferred embodiment of the invention and wherein:

Figure 1 shows body weights of animals as a percentage of starting weight after a single subcutaneous injection of saline or CHB (n=4, results expressed as means ± SEM.) over time after treatment;

Figure 2 shows pancreatic weight as a percentage of body weight in animals after a single subcutaneous injection of saline or CHB (n=4, results expressed as means ± SEM.) over time after treatment;

Figure 3 shows pancreatic morphology after a single subcutaneous injection of saline of CHB. All H and E stained. **(A)** 48 hours after saline. There is wide separation of ducts (arrows) and islets (I) by closely-packed acinar cells (x500). **(B)** 12 hours after CHB. Note numerous apoptotic acinar cells with characteristic nuclear morphology (arrow) (x1200). **(C)** 18 hours after CHB. Most acinar cells contain pyknotic nuclear remnants (arrowheads) and show cytoplasmic swelling and vacuolation; a few appear normal (arrows) (x900). **(D)** 48 hours after CHB. Advanced secondary necrosis affecting all acinar cells in field. Intact duct is indicated by arrow (x360). **(E)** 96 hours after CHB. No acinar cells remain. Atrophic lobules comprise crowded ducts in a connective tissue stroma. (x200). **(F)** 28 days after CHB. Sparse regenerative acini are seen adjacent to islets (arrows). Note few ducts in a collagenous stroma and prominent fatty infiltration, (x200);

Figure 4 shows pancreatic immunohistochemistry after a single subcutaneous injection of saline or CHB. All with haematoxylin counterstain. **(A)** 24 hours after saline. Widely-spaced keratin-positive ducts (arrows) are separated by closely-

packed keratin negative acinar cells (x160) **(B)** 48 hours after CHB. Widely-spaced keratin-positive ducts (arrows) separated by keratin-negative nonviable acinar cells (x180). **(C)** 96 hours after CHB. Lobules comprise crowded keratin-positive ducts separated by loose connective tissue. No acinar cells are seen, (x180). **(D)** 96 hours after CHB. Ducts are negative for amylase. Note isolated amylase positive epithelial cell (arrow) and perinsular amylase positivity (x160);

Figure 5 shows pancreatic ultrastructure after a single subcutaneous injection of CHB. **(A)** 12 hours after CHB. Adjacent apoptotic acinar cells show well-demarcated crescentic clumped chromatin, large nucleolar remnants (Arrowhead) and whorling of endoplasmic reticulin (arrows) (x3000). **(B)** 18 hours after CHB. Apoptotic acinar cells show nuclear fragments with crescentic clumped chromatin but dilation of endoplasmic reticulin, swollen mitochondria (arrowheads) and plasma membrane rupture (arrow). Contrast with adjacent viable acinar cells (x2800). **(C)** 48 hours after CHB. Note viable duct epithelial cells (D), residual acinar cell cytoplasmic debris (A) and intra-acinar macrophage (M) laden with residual bodies. The pale cell in the duct epithelium (arrow) is also likely to represent an intraepithelial macrophage (x350). **(D)** 96 hours after CHB. Duct with typical indented nuclei and sparse organelles of lining cells. Note mitotic lining cell (M), intraepithelial apoptotic body (arrow), and adjacent collapsed redundant basement membrane (arrowhead) (x2500). **(E)** 96 hours after CHB. Activated and mitotic interstitial fibroblasts. (x.3300). **(F)** 48 hours after CHB. Capillary with intraluminal apoptotic bodies of presumed endothelial cell origin (arrow). Note adjacent intraacinar macrophage (M) with residual body-laden cytoplasm (x.7000);

Figure 6 shows **(A)**. Untreated AR42J acinar cell carcinoma in an athymic rat, showing broad sheets of cells with an area of haemorrhagic necrosis (arrow) (x100).

(B) Untreated AR42J acinar cell carcinoma, (x400), showing multiple mitoses. (C) AR42J acinar cell carcinoma 24 hours after a single subcutaneous dose of 140mg/kg CHB, showing widespread apoptosis and an area of surviving cells (arrow). (x100). (D) AR42J acinar cell carcinoma 24 hours after a single subcutaneous dose of 140mg/kg CHB, showing widespread apoptotic change (x400).

Figure 7 shows (A) Untreated AR42J acinar cell carcinoma, showing primitive acini formation (x1000). (B) AR42J acinar cell carcinoma 24 hours after a single subcutaneous dose of 140mg/kg CHB, showing pyknotic and fragmented nuclei and secondary necrosis of cytoplasm (x1000). (C) Pancreas of a normal Wistar rat 24 hours after 140mg/kg subcutaneous CHB showing almost total acinar cell death via apoptosis with secondary necrosis (x400). (D) Pancreas of athymic rat 24 hours after 140mg/kg subcutaneous CHB showing regional effect with necrotic areas adjacent to well-preserved areas with pyknotic nuclei (x400).

Fig.8 shows an electronmicrograph of apoptotic cells showing condensed marginated chromatin.

Experimental

1. Determination of Acinar Cell Apoptosis and Regeneration of Acinar Cells with Subcutaneous Administration of CHB.

1.1 Material and Methods

Synthetic CHB made according to the method of Das and Torssell (Das and Torssell 1983 Tetrahedron 39:2243-2247).

Male Wistar rats weighing 200 – 250 g were caged in pairs and given food and water ad libitum with a 12 hours light-dark cycle. Twelve groups of 10 rats were

divided randomly into 6 test animals and 4 control animals. At time 0, test animals were given 150mg/kg of CHB mixed in 0.5ml sterile normal saline and controls were given 0.5ml sterile normal saline subcutaneously.

For light microscopy, 4 experimental and 4 control animals were killed at 2, 4, 6, 12, 24, 48, 72 and 96 hours and 7, 10, 18 and 28 days using 60 mg intraperitoneal pentobarbitone. Animals were weighed and the pancreas removed, weighed and processed using routine methods. Additional pairs of experimental animals were killed at 18 and 60 hours for electron microscopy and morphological study. Weights were recorded as means ± standard error of the mean (SEM). Differences between means were analysed using Student's t-test.

For quantification of apoptosis, apoptotic cells and bodies, identified using the morphological criteria (Kerr et al. 1995 Method Cell Biol. 46:1-27) and were counted in ten high-power graticule fields (HPF), selected at random, in a histological slide from each animal at 2, 4, 6 and 12 hours with the proviso that mostly acinar tissue filled the field. A group of tightly clustered apoptotic bodies, presumably derived from a single cell, was recorded as a single count. An estimate of the total number of acinar cells per HPF in each slide was made for calculation of an apoptotic index (apoptotic count as a percentage of total acinar cells present). Counts/HPF and apoptotic indices were recorded as means ± SEM for each group. Differences between means were analysed using Student's t-test. Terminal d-UTP nick-end labelling (TUNEL) was not used because it is our experience and the experience of others that it is not always specified for apoptosis, and ultimately, apoptosis must be confirmed morphologically (Ansari et al. 1993 J. Pathol. 170:1-8; Grasl-Kraupp et al. 1995 Hepatology 21:1465-1468).

Immunohistochemistry for cytokeratin an amylase was performed to identify cells in sections as duct (Schussler et al. 1992 Am. J. Pathol 140:559-568; Bouwens et al. 1995 J Histochem Cytochem. 43:245-253) or acinar (Bendayan 1984 Histochem J. 16:85-108) respectively. For cytokeratin, deparaffinized sections were pretreated with 0.1% trypsin, then 0.3% hydrogen peroxide in methanol followed by mouse monoclonal AE1/AE3 anti-cytokeratin at a dilution of 1/40. Secondary antibody was rat anti-mouse biotinylated IgG used at a dilution of 1/400. Antibody-binding was demonstrated using the peroxidase-labelled streptavidin biotin complex method and reactions were developed with 3,3'-diaminobenzidine tetrahydrochloride solution. For amylase, deparaffinized sections were boiled in Target Retrieval Solution then placed in 0.3% hydrogen peroxide in methanol. Primary antibody was anti-rabbit immunoglobulin used at a dilution of 1/500 and secondary antibody was anti-rabbit goat biotinylated IgG used at a dilution of 1/400. Antigen-binding was demonstrated using the peroxidase-streptavidin method developed with Vector VIP peroxidase substrate. All sections were lightly counterstained with hematoxylin.

For electron microscopy, two rats from each test group were deeply anaesthetised with intraperitoneal sodium pentobarbitone. A catheter was inserted into the abdominal aorta and the vasculature flushed in sequence with 1) heparinized normal saline, 2) 1% paraformaldehyde and 1.2% glutaraldehyde in cacodylate buffer and 3) 4% paraformaldehyde and 5% glutaraldehyde in cacodylate buffer (Karnovsky 1965 J, Cell Biol.27:137A-138A). Pancreas was removed immediately, diced and immersed in perfusate no. 3 for two hours, then stored in cacodylate buffer. The tissue was postfixed in 1% osmium tetroxide, stained en bloc in 5% aqueous uranyl acetate, dehydrated through a series of graded alcohols, cleared in propylene oxide, and embedded in an epon-araldite mixture. Semithin sections (1μm) were cut on an

LKB Ultratome V and stained with toluidine blue for viewing. Ultrathin sections from selected areas were picked up on uncoated copper grids, stained with lead citrate and examined with a JEOL-1200 EX11 electron microscope.

1.2 Results

1.2.1 General Observations

Control rats showed no behavioural change and normal weight gain reaching 180% at 28-days (Figure 1). Experimental rats showed discomfort 30 minutes after injection, lost curiosity and became reluctant to move. Body weight fell over the first week and thereafter remained unchanged (Figure 1).

At autopsy, control rats had normal viscera and a pancreatic weight which was constant as a proportion of body weight (Figure 2). Experimental animals showed pancreatic edema from 2 hours, actual pancreatic weight reaching 4.41±0.71 g at 6 hours (compared to 0.78±0.14g in controls, $P<0.001$), then falling. Atrophy was apparent at 7 days and persisted, actual pancreatic weight falling to 0.44 ±0.04g at this time compared to 1.80±0.08g in controls, $P<0.001$.

Changes in pancreatic weight as a percentage of body weight are shown in Figure 2. At 18 and 28 days CHB-treated rats had muscle wasting, abdominal distension and dilated bowel containing undigested food.

1.2.2 Light Microscopy

Control animals showed histologically normal pancreas (Figure 3A). From 2 hours test animals showed mild dilation of acinar lumens and acinar cell vacuolation and depletion of zymogen granules.

Apoptotic acinar cells, evident at 6 hours, showed sharply-defined crescents of clumped chromatin against the nuclear envelope but infrequent fragmentation. Their number reached 178 ±10/HPF at 12 hours (compared to 0.85 ±0.13/HPF in controls, $P<0.001$) or 23.6±7.43% of acinar cells (compared to 0.001% in controls) (Figure 3 B). By 18 hours most acinar cells had chromatin changes of apoptosis but swollen vacuolated cytoplasm indicative of "secondary necrosis" (Figure 3C) which subsequently progressed (Figure 3D). By 96 hours no acinar cells remained (Figure 3E). A few regenerative acini appeared by 18 days, particularly adjacent to islets of Langerhans, but thereafter they did not increase appreciably in number (Figure 3F).

Intercalated ducts were mildly dilated at 4 hours, duct cell mitoses were prominent at 48 hours, and at 96 hours, lobules comprised groups of ducts within a connective tissue stroma (Figure 3E). Small numbers of apoptotic bodies continued to be seen within duct lumens and epithelium. By 7 days ducts had larger lumens and flattened lining epithelial cells. Thereafter the number of ducts decreased with few remaining at 18 and 28 days (Figure 3F).

Interlobular edema was present from 2 hours and interlobular edema from 4 hours; both persisted for 72 hours. The interstitial spaces were acellular before small numbers of mononuclear phagocytes appeared about vessels at 4 hours and within acini at 24 hours. They reached moderate numbers at 48 hours, peaked at 72 hours, then declined markedly by 7 days. Sparse neutrophils were present from 12 hours and mitotic mononuclear phagocytes at 48 hours.

Enlarged mitotically active fibroblasts were seen 48 hours, by 96 hours fibroblasts and collagen enveloped lobules and at 7 days fibroblasts were less prominent and collagen was found both in and around lobules. At 28 days the pancreas comprised largely fat, collagen and islets (Figure 3F).

Islets were not studied in detail. Given the degree of atrophy, however, less islet tissue was apparent than might be expected from simple condensation.

1.2.3 Immunohistochemistry

In controls ducts were positive for cytokeratin and acinar cells negative (figure 4A). At 48 hours in test animals, when few viable acinar cells remained, cytokeratin marked dispersed intact ducts and duct cells (Figure 4B). At 96 hours ducts of atrophic lobules, the only remaining epithelium, were positive for cytokeratin (Figure 4C). Amylase was demonstrated in apoptotic cells at 18 hours, confirming their acinar cell origin. There were no or rare amylase-containing cells at 72 and 96 hours (Figure 4D) with occasional apoptotic bodies staining for amylase. The periphery of islets also showed amylase staining at 96 hours (Figure 4D).

1.2.4 Electron Microscopy

Controls showed normal pancreatic ultrastructure (Ekholm et al. 1962 J. Ultrastruct. Res. 7:61-72; Ekholm et al. 1962 7:73-83). In test animals acinar cell apoptosis was slightly increased at 6 hours and markedly increased at 12 hours, when large numbers of adjacent cells were often affected (Figure 5A). Apoptotic cells showed sharply-defined crescents of chromatin abutting the nuclear envelope, prominent nuclear remnants, whorling of endoplasmic reticulum and structural preservation of organelles (figure 5A) but cellular fragmentation to form apoptotic bodies was uncommon. At 18 hours, apoptotic cells, identified by their nuclear characteristics, remained in situ, but showed dilation of endoplasmic reticulum and nuclear envelopes, swelling and rupture of mitochondria and rupture of plasma membranes (Figure 5 B), so-called "secondary necrosis". This process progressed

such that, by 48 hours, acinar cells were reduced to degraded cellular material associated with small number of intraepithelial macrophages containing ingested apoptotic bodies, degraded cellular material in phagosomes of residual bodies (Figure 5C). By 96 hours, acinar cell debris had been removed (Figure 5D). Ducts and duct cells survived, showing increased mitotic activity, particularly at 60 and 72 hours (Figures 5C and D). Small numbers of ductal intraepithelial apoptotic bodies and surrounding collapsed basement membrane were identified (Figure 5 D).

From 48 to 96 hours, prominent activated and mitotic fibroblasts were seen (Figure 5E). At first collagen was sparse but increased in amount towards 7 days. At 48 hours, mitoses in interstitial macrophages were confirmed and endothelial cell apoptosis was present in interstitial capillaries (Figure 5F); this continued over succeeding days. By 18 days, isolated regenerative acini comprised acinar cells closely resembling acinar cells in control glands.

1.3 Discussion

Within 12 hours of administration of CHB, there is relatively synchronous onset of apoptosis in the majority of acinar cells. This contrasts with the slow onset of apoptosis and gradual increase peaking about the third day that occurs after duct ligation (Walker 1987 Am. J. Pathol. 126:439-451) or the administration of cerulein (Fujimoto et al. 1997 Digestion 58:421-430) or ethionine and a protein-depleted diet (Walker et al. 1993 Pancreas 8:443-449). The sequence is similar but delayed after administration of a copper-depleted diet (Rao et al. 1993. Am. J. Path 142:1952-1957).

The rapid and synchronous onset of apoptosis after CHB administration overwhelms the capacity of duct cells, viable acinar cells and tissue macrophages to rapidly remove apoptotic cells. As a consequence, most of the apoptotic cells remain in situ undergoing progressive swelling, rupture of organelle and plasma membranes and degradative change referred to as "secondary necrosis".

A feature of the CHB model of pancreatic involution is the limited fragmentation of apoptotic acinar cells compared with that seen, for example, after duct ligation (Walker 1987 supra). In the first hours after CHB administration and at lower doses, apoptosis proceeds to cell fragmentation with intraepithelial macrophages at 12 hours engorged with phagocytosed apoptotic bodies making it unlikely that CHB prevents microfilament reorganisation.

Despite early cell death and edema, inflammatory cell infiltration is delayed, reaching moderate density only at 48 hours, the number of neutrophils remaining small throughout. In contrast, cerulein excites a vigorous inflammatory response (Walker et al. 1993 supra; Fujimoto et al 1997 supra).

Acinar cell regeneration is limited to a few acini 10-18 days after CHB administration. After cerulein and ethinione administration, it is rapid and complete once the causative agent is removed (Fitzgerald 1960 Lb. Invest. 9:67-85; Isasser et al. 1986 Pancreas 1:421-429).

2. Determination of Differences in Effect with Oral and Subcutaneous Administration

2.1 Method..

Eight groups of eight male Wistar rats of approximately 200g were divided into 6 test animals and 2 controls. Control animals were given either water orally by gavage or

saline subcutaneously as appropriate to their group. Test animals in each group were given CHB daily for 4 days, with doses for groups 50, 100, 150 and 200mg, orally by gavage in water or subcutaneously in saline. A further group was treated later with 130mg/kg subcutaneously in order to further define the dose-effect. Animals were weighed each day and the day's dose of CHB calculated accordingly. Twenty-four hours after the fourth dose animals were euthanased using 60mg intra-peritoneal pentobarbitone and a full autopsy performed. Organs were removed and weighed before routine processing for histological examination. These were pancreas, brain, thymus, lungs, heart, salivary glands, lumbar vertebrum, paraspinal skeletal muscle, pancreas, liver, intestine, kidney, spleen, seminal vesicle, testicle and prostate.

2.2 Results:

General

Three rats receiving 200mg/kg/day orally died after two doses, the remaining three test and two control animals were euthanased the same day. Three rats receiving 200mglkg/day subcutaneously were dead after one dose, necessitating the euthanasia of the rest of the group. One rat in the 100mg/kg oral test group died after one dose. Overall body weight gain was reduced for all test animals, those animals receiving 130, 1 50 and 200mg/kg/day losing weight daily. Control animals gained 5 grams per day, rats given 50 and 100mg/kg/day gained 1-4 grams per day, rats given 130, 150 and 200 mg/kg/day lost weight. Pancreas and liver weights were not recorded. Spleen, thymus and lung weight was reduced in test animals, more so in high-dose and subcutaneous groups. Kidney weight was unchanged and testicle weight as a proportion of body weight rose.

In the 200mg/kg s/c rats found dead there was haemorrhagic change in liver, bowel, and under claws, pancreatic swelling and erythema, fat necrosis in the abdomen and ascites. One rat in the 150mg/kg oral group had liver infarction and bleeding in the thoracic and abdominal cavities.

Pancreas

The effect of CHB on the pancreas was found to be dependent on both dose and route. As with bodyweight, the subcutaneous route has a stronger effect. There was considerable variability among the rats and within each pancreas.

Oral administration: Rats given 200mg/kg showed edema and moderate apoptosis at 24 hours after their second dose. Other groups were examined 24 hours after the fourth daily dose. The 150mg/kg group showed less effect with mild to moderate apoptosis, and occasional mitosis of acinar cells. With 100mg/kg, there was mild apoptosis of acinar cells but conspicuously increased mitotic activity. The same was true of the 50mg/kg group, with apoptosis less obvious.

Subcutaneous administration: Rats given CHB subcutaneously showed a greater effect on the pancreas for the same dose. In the group given 200mg/kg, the three rats which were dead at 24 hours all had marked Pancreatic edema and moderate apoptosis. The pancreatic lobules were intact. In the three survivors which were euthanased at 24 hours there was apoptosis of approximately 80% of acinar cells with progression to secondary necrosis. In the 150mg/kg group, the effect 24 hours after 4 daily doses was almost complete loss of acinar cells, with lobules composed of ductal complexes, mononuclear inflammatory cells and islets. Both apoptosis and mitosis was present in ductal complexes and perilobular fibrosis was present. In four of the six rats a layer of cells was present around the outer edge of

islets. At 130mg/kg, the same process of acinar cell loss occurred but was less complete, about one-third of acinar cells surviving, and mitosis of acinar cells was seen. Inflammation and mononuclear cell infiltrate were as marked as at the higher doses. Peri-insular ductal cells were also present at this dose. At 100mg/kg, acinar cell loss was about 20 – 80% with greater variability between rats and within pancreases. 50mglkg caused only mild apoptosis but mitosis was increased.

Other Organs

Of other organs examined only the liver showed significant changes. The effect on the liver was greater when CHB was given by the oral route.

Oral CHB: In the three rats that died after 200mg/kg all had widespread necrosis of the liver. Nuclear changes were those of apoptosis with chromatin condensation and fragmentation. Hepatocytes were necrotic and there were patches of haemorrhage. In the three rats that survived until euthanasia, one rat had the same degree of liver damage, another had focal inflammatory foci and loss of hepatocytes, another had fine vacuolation of most hepatocytes with apoptotic bodies in sinusoids.

150mg/kg orally induced liver changes in half the rats with focal loss of hepatocytes, necrotic patches and clusters of sinusoidal cells in portal areas of the lobule. Lower doses had no discernible effect.

Subcutaneous CHB: Four daily doses of 150mg/kg induced changes of liver necrosis in subcapsular areas in contact with pancreas. The remainder of these livers were normal.

2.3 Discussion

The effect of CHB on the pancreatic acinar cell is dose-related. The lesser effect of the same dose by oral route is not surprising, attributable to less complete absorption from the GIT and/or metabolism and neutralization in the liver as a first-pass effect. When 200mg/kg is given orally in water it has a more toxic effect than when in a corn-oil vehicle, causing fatal liver necrosis in half the animals and severe hepatocyte damage in the survivors. Even at 150mg/kg orally, liver damage was significant with only mild-moderate pancreatotoxicity. It is expected that absorption of a water solution would be more complete and rapid than that of a suspension in oil. Liver necrosis was evenly widespread. In the group given 150mg/kg subcutaneously, liver necrosis was confined to subcapsular areas in contact with pancreas, suggesting a local effect possibly due to enzyme action. The effect on the pancreas of this hepatotoxic-dose is moderate. In a study of the pancreatotoxic effects of CHB given in three vehicles (Wallig et al, 1989), the liver was not studied. If CHB is to be used for its pancreatotoxic effects, then the oral route should be avoided.

3. *In Vivo* effect of CHB on Pancreatic Carcinoma

3.1 Method

Athymic rats 200 –250g were purchased from the Animal Resources Centre, Western Australia. Four experiments were performed using slightly different doses with the intention using a dose just sub-lethal in order to assess maximal effect.

Experiment 1: *Ductal* carcinoma cells (2×10^6 cells of Capan 2) were injected into the left flank of 7 nude rats. Sixty days later, tumour nodules were 1 –2cm diameter. By the time tumours were ready for testing, rats weighed approximately 300g. 135mg/kg CHB (absolute dose 38 - 40mg) was given into ventral abdominal subcutaneous tissue in 0.5ml sterile saline with 2 controls getting saline only. At 18

hours, all test rats were dead. Autopsies were performed taking specimens of carcinoma, pancreas and liver for processing.

Experiment 2: 6 rats were inoculated in the right flank with 5 x 10^6 cells of Ar42J rat *acinar* cell carcinoma. By 13 days all grew tumours 1 – 2cm diameter. CHB at a dose of 125mg/kg mixed in 0.5ml saline (absolute dose 26 – 32.5mg) was injected into ventral abdominal skin of all 6 rats. In this experiment there were no controls. All rats survived until eighteen hours later when they were euthanased using 60mg intraperitoneal pentabarbitone. Carcinoma, pancreas and liver were dissected out and placed in formalin for processing.

Experiment 3: 14 rats were inoculated with 5 x 10^6cells of Ar42J *acinar* cell carcinoma. Seven rats were given CHB at 140mg/kg (approximately 30 – 32mg absolute) and another 7 rats were given saline only. At 18 hours all rats had survived and were euthanased, pancreas, liver and carcinoma removed and taken for processing.

3.2 Results

Experiment 1: *Ductal* carcinoma nodules were not effected by CHB. Histological appearance was the same in both test and control groups.

Experiment 2: At lower doses of CHB, 1 of 6 test rats had evidence of a cytotoxic effect with widespread apoptosis and secondary necrosis. Apoptosis was as described previously, with crescentic clumping of chromatin, as well as wheel-rim clumping around the nuclear membrane. Fragmentation of cells was not obvious as

described in other settings of massive synchronous apoptosis. In order to distinguish the effect of CHB from patchy haemorrhagic necrosis, an effect was regarded as present only if little viable tumour remained.

Experiment 3: Control tumour nodules were composed of sheets of uniform large cells with dilated vascular channels and some areas of haemorrhage and necrosis (Figure 6A and B, Figure 7A). Patches of apoptotic cells were present in places, particularly near haemorrhages. Mitotic rate varied but in places was very high. Three of 7 rats had a marked effect (Figure 6C and D, Figure 7B), a further 2 had about half surviving and the remaining 2 rats had no discernible effect.

Three test rats with acinar cell carcinoma that died of CHB toxicity in preliminary dose-testing experiments had almost total cell death in the tumour nodules. The fact that one rat had some patches of surviving tumour cells makes post-mortem change unlikely to be responsible for the apoptotic appearance.

The effect on pancreas was different in athymic and Wistar rats (Figure 7C and D). Athymic rats had a regional effect in the pancreas with areas of secondary necrosis juxtaposed with well-preserved areas of acinar tissue, albeit with pyknotic nuclei.

3.3 Discussion:

There is no discernible effect of CHB on ductal cells in the normal pancreas. It is therefore not surprising that malignant ductal cells are not substantially affected by CHB. Normal acinar cells, however, are sensitive and can be eliminated by a single subcutaneous dose of 140mg/kg in the Wistar rat. Only very limited regeneration of acinar cells was seen at 28 days, and this was in the peri-islet areas.

The usual picture of apoptosis is that nuclear changes are followed rapidly by cytoplasmic condensation, blebbing and fragmentation. As described in other settings of massive synchronous apoptosis fragmentation was not obvious.

In the nude rat tumour nodules the effect of CHB appeared to be total regional apoptosis or nothing. No part effect was seen. At the lower dose, 1 of 6 rats had an almost total cell kill, 5 had no discernible effect. At the higher dose, 3 of 7 had a marked effect with few areas of viable cells, and a further 2 of 7 had apoptotic change in about half the tumour section. In unaffected tumours, there was no increase in apoptosis and no decrease in mitosis. Areas of tumour were either totally apoptotic or seemingly unaffected.

The effect on pancreas in the athymic rat is different from that in the Wistar rat. Despite marked edema as expected, the appearance of the pancreas is unusual in that nuclei in both necrotic and well-preserved areas are pyknotic. Patches of apoptotic cells are seen in the liver. It may be that a thymic humoral component is involved in the widespread apoptotic process in Wistar pancreas, without which the lesion is different.

An advantage of the current invention is that the method of treatment can be used to kill acinar carcinoma cells in the pancreas and spread throughout the body. Acinar carcinoma cells are resistant to radiation treatment and chemotherapy. While it may be possible to surgically remove the pancreas and hence the acinar carcinoma cells in the pancreas, detection of acinar cell carcinoma usually only occurs after the acinar carcinoma cells have spread from the pancreas. Furthermore the treatment only killed the acinar cells, and other cells in the pancreas appeared to be biologically functional after the treatment. Thus a patient would possibly avoid becoming diabetic

with functionally active islet cells in the pancreas. With surgical removal of the pancreas a patient becomes diabetic and insulin must be administered.

It will of course be realised that while the foregoing has been given by way of illustrative example of this invention all such and other modifications and variations thereto as would be apparent to persons skilled in the art are deemed to fall within the broad scope and ambit of this invention as defined in the claims appended hereto.

CLAIMS:

1. A method of providing selective, substantially total, non-regenerative apoptosis of pancreatic acinar cells comprising a single-dose, subcutaneous or intra-arterial administration of a composition of cyanohydroxybutene and a pharmacologically acceptable aqueous carrier.

2. A method according to claim 1, wherein said therapeutic window is selected to minimise liver damage in said patient.

3. A method according to claim 1 or 2, wherein said administration is subcutaneous.

4. A method according to any one of claims 1 and 3, wherein said cyanohydroxybutene is administered at a dosage within the range of 140-160 mg CHB/kg of body weight.

5. A method according to any one of claims 1 to 4, wherein said patient is selected on the basis of said pancreatic acinar cells including acinar carcimoma cells.

6. A method for treating pancreatic disease including administering to a patient a single-dose, subcutaneous or intra-arterial, therapeutically effective amount of cyanohydroxybutene wherein said amount is sufficient to cause selective, substantially total, substantially non-regenerative apoptosis of acinar cells in the patient.

7. A method of treating a subject having a pancreatic carcinoma involving acinar cells and including the steps of:

preparing a cyanohydroxybutene (CHB) formulation; and

administering subcutaneous or intra-arterial single dose of a CHB formulation to said subject in an amount sufficient to cause selective, substantially total, substantially non-regenerative apoptosis of malignant acinar cells in a patient.

8. A method as claimed in claim 7 wherein the CHB dose is within a range of 125-160 mg CHB/kg of body weight.

9. A method as claimed in claim 8 wherein the CHB dose is within the range of 140-160 mg CHB/kg of body weight.

10. A method as claimed in claim 7 wherein the carcinoma involves either acinar cell carcinoma or pancreatic carcinoma containing a mixed population of cells including acinar cells.

11. A method as claimed in claim 7 wherein said CHB molecule is conjugated to a ligand which is selected to to bind to an acinar cell surface receptor.

12. A method according to any one of claims 7 to 11, wherein said dose is selected whereby liver damage in the subject is minimised.

13. A method of treating acute or chronic pancreatitis including the steps of:

preparing a cyanohydroxybutene (CHB) formulation; and

administering a subcutaneous or intra-arterial single dose of a CHB formulation to said subject in an amount sufficient to cause selective, substantially total, substantially non-regenerative apoptosis of malignant acinar cells in a patient.

14. A method of treating acute or chronic pancreatitis as claimed in claim 13 wherein the CHB dose is within a range of 125-160 mg CHB/kg of body weight.

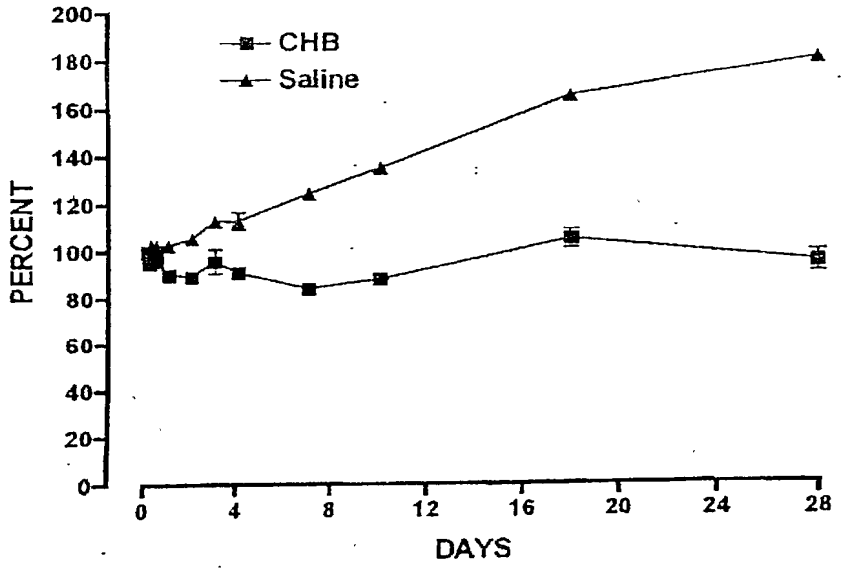
15. A method of treating acute or chronic pancreatitis as claimed in claim 13 or 14 wherein the CHB formulation is administered by subcutaneous injection.

16. A method according to any one of claims 13 to 15, wherein said dose is selected whereby liver damage in the subject is minimised

ABSTRACT

TREATMENT OF PANCREATIC DISEASE

The present invention relates to the administration of cyanohydroxybutene (CHB) to eliminate acinar cells in a subject. Subcutaneous injection of CHB at a sub-lethal dosage casued apoptosis of the substantially entire population of acinar cells. The pancreatic lesion has marked early edema with limited inflammatory infiltration, rapid synchronous onset of acinar cell apoptosis and advanced atrophy with a severely limited regenerative response. There is further provided methods of treatment of acinar cell carcinoma and pancreatitis.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PERCENT
200
180
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120
100
80
60
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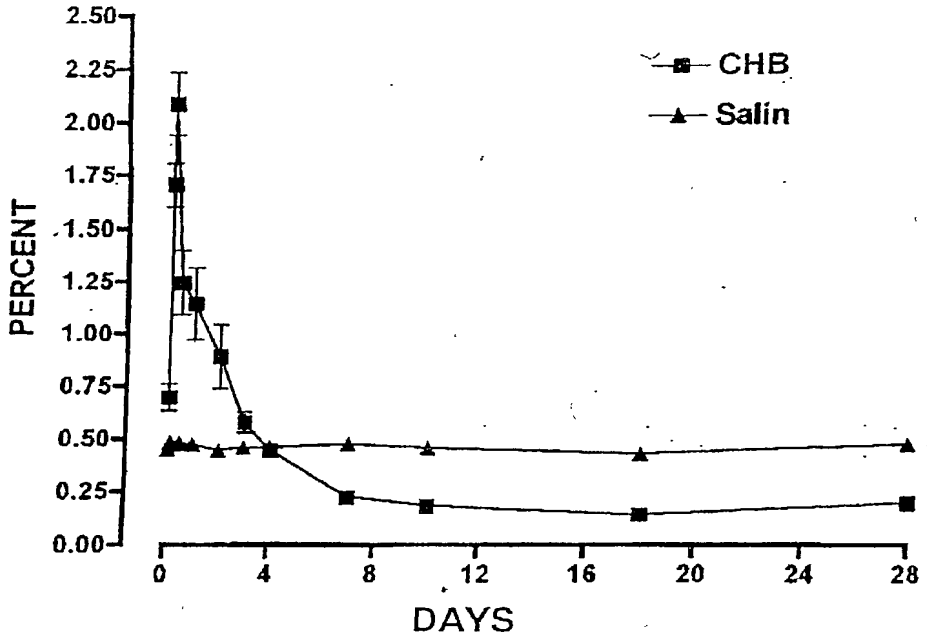
0
4
8
12
16
20
24
28
DAYS

Figure 1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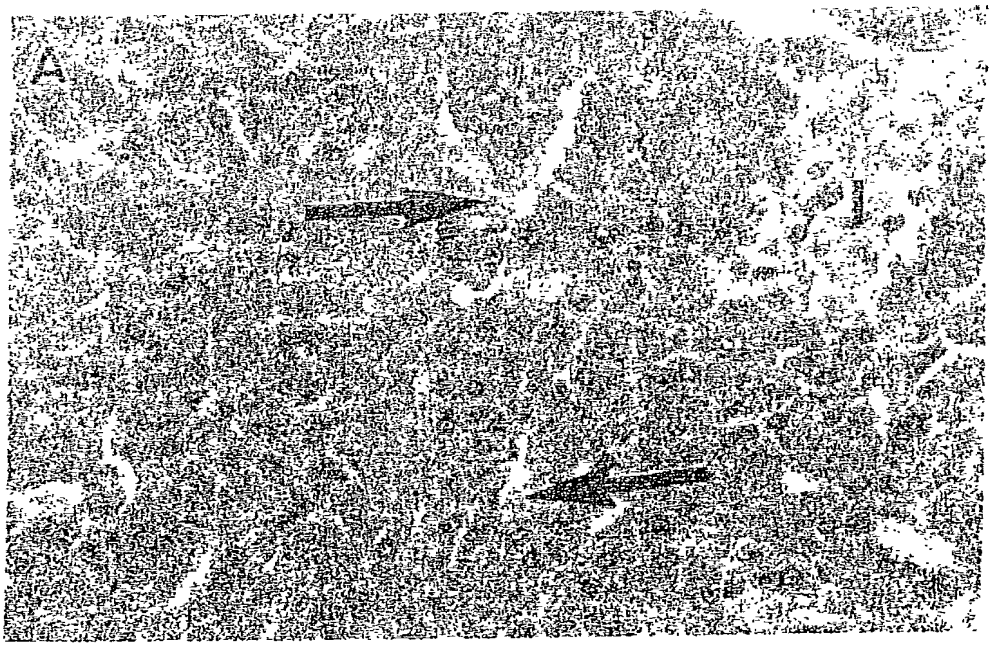
DAYS
CHB
Salin

Figure 2



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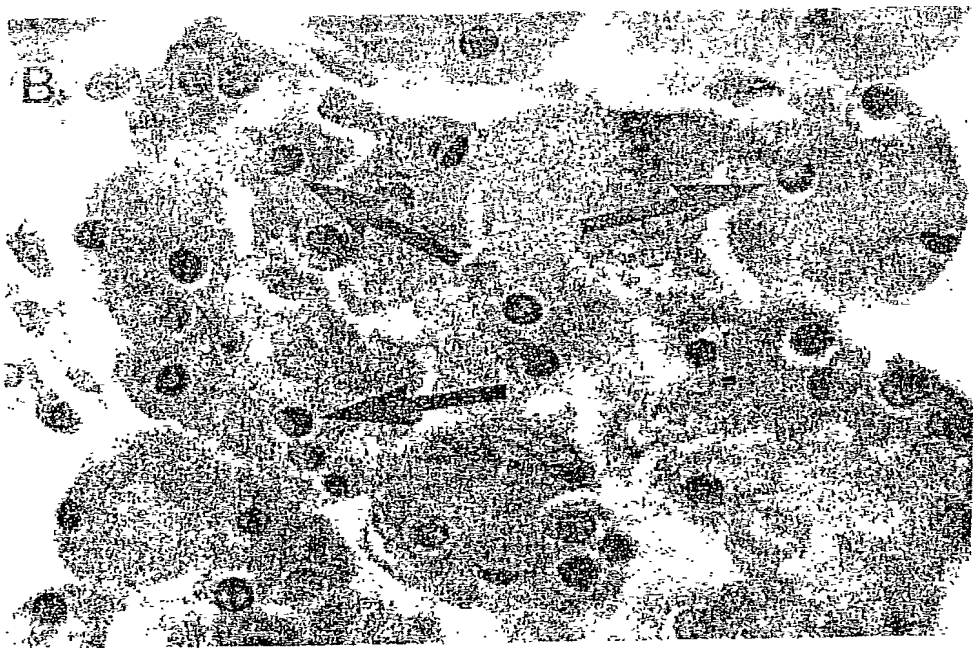


Figure 3B

Figure 3C

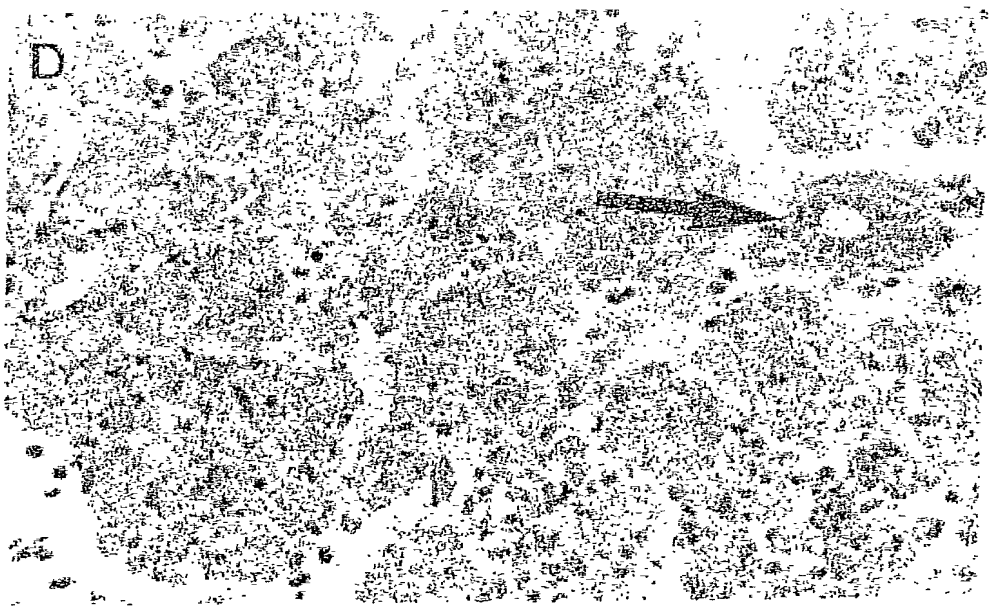


Figure 3D

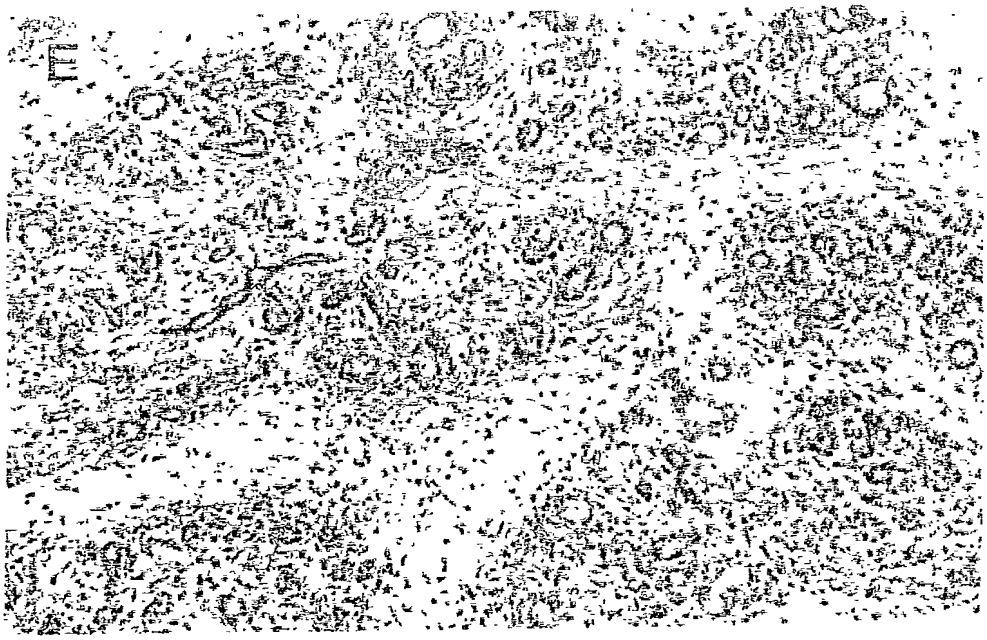
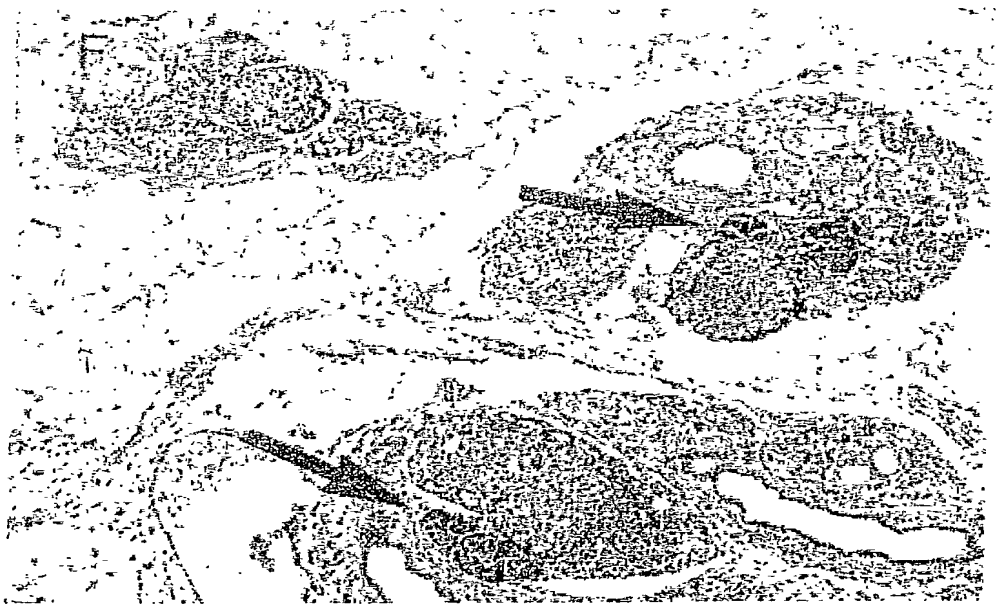


Figure 3E



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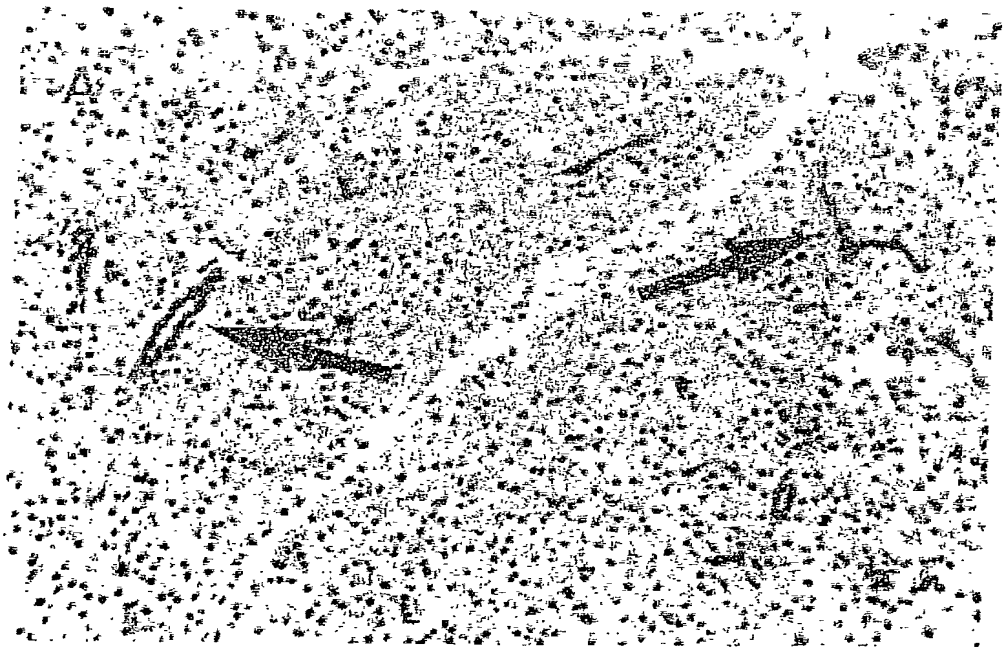

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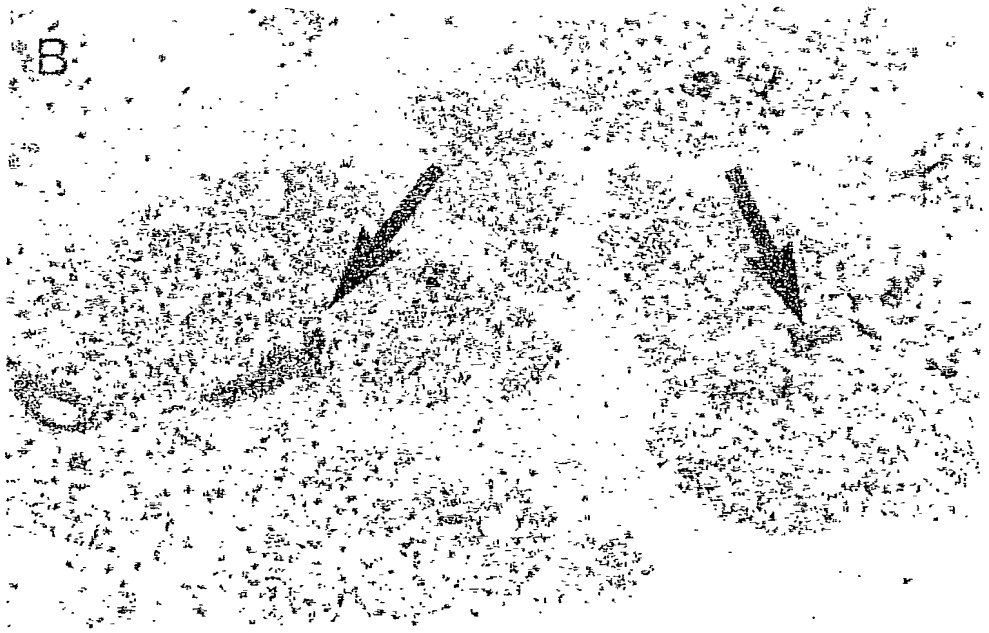
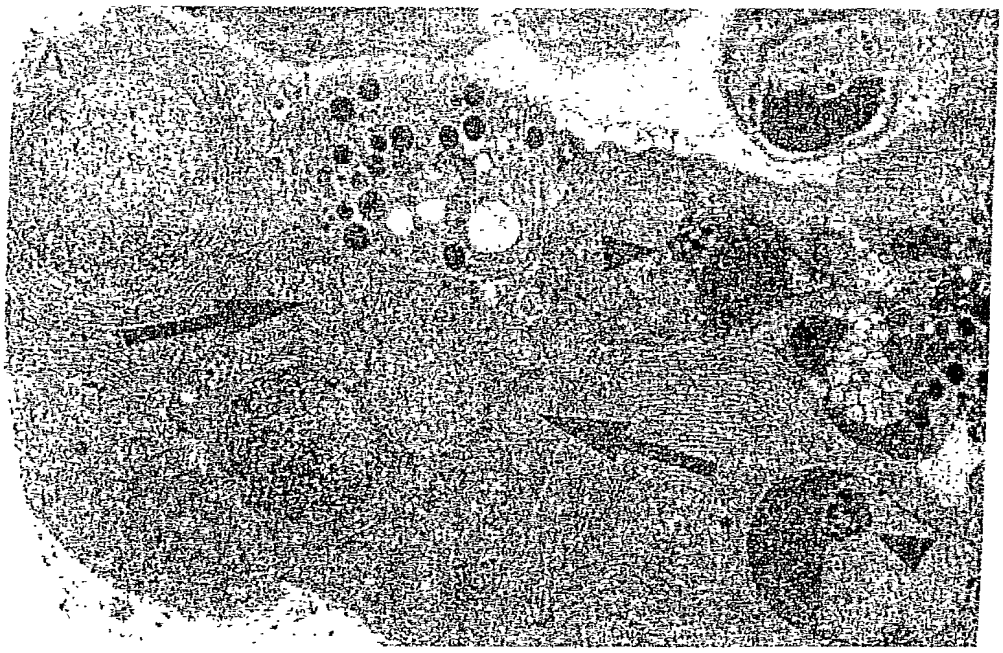


Figure 4B

Figure 4C

Figure 4D



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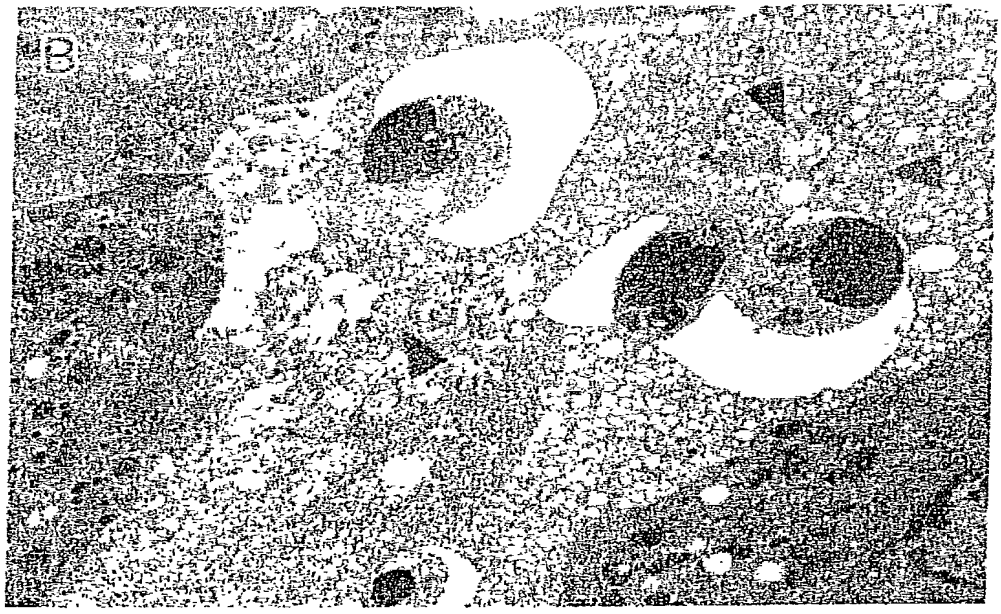

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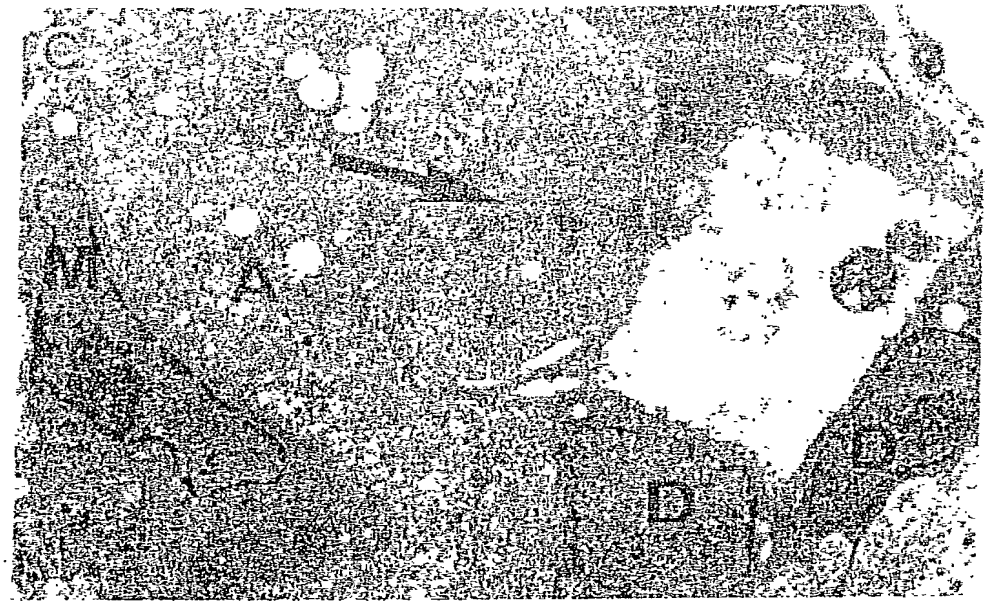

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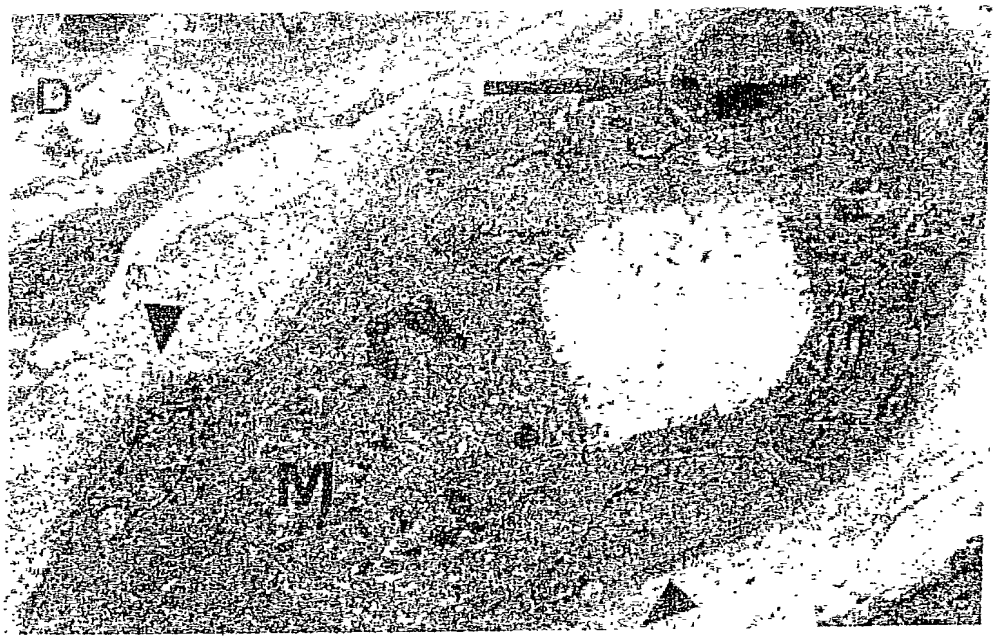
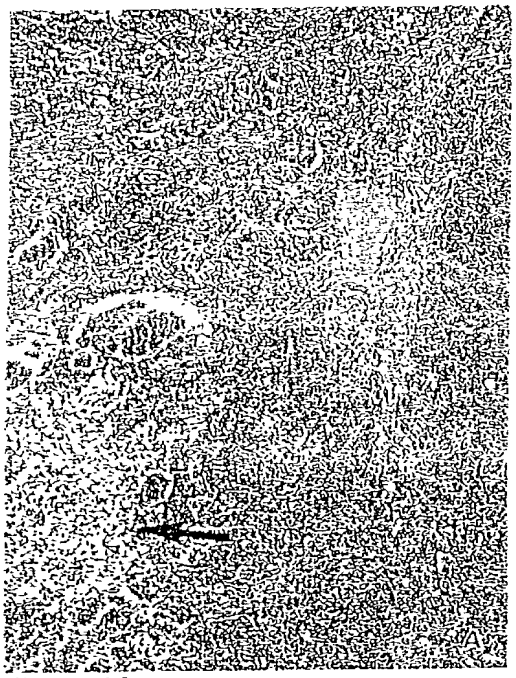


Figure 5D

Figure 5E

Figure 5F



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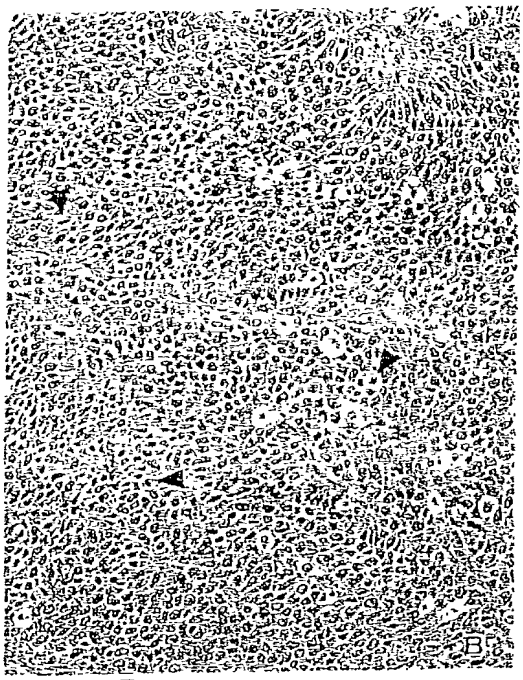

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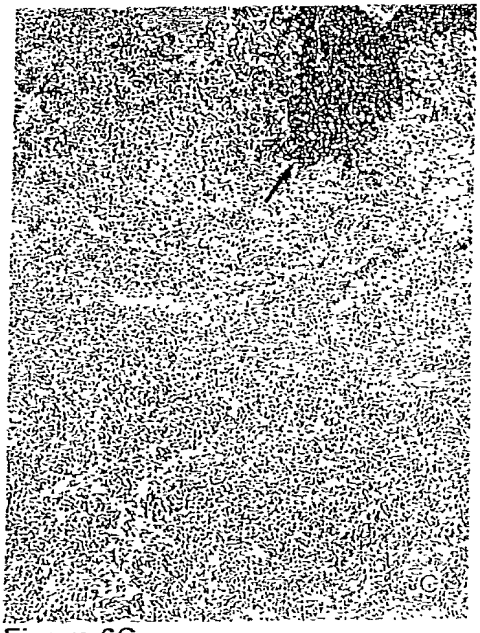

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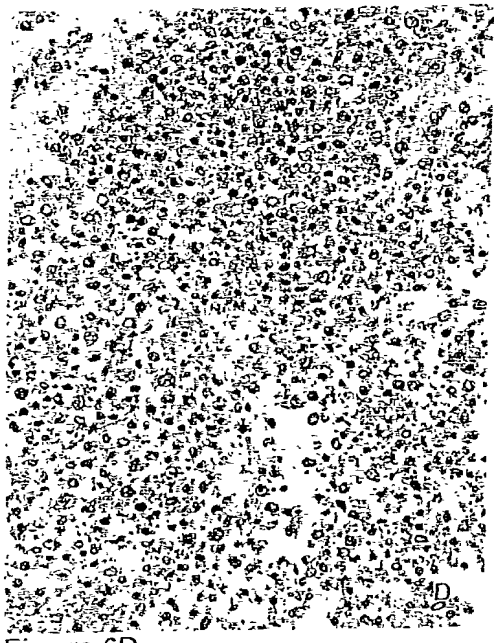

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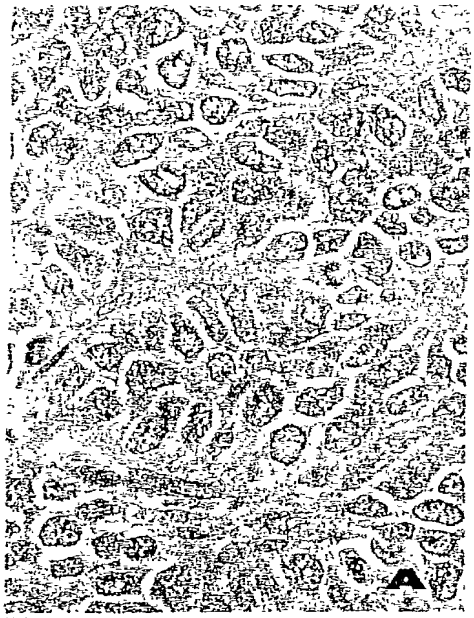

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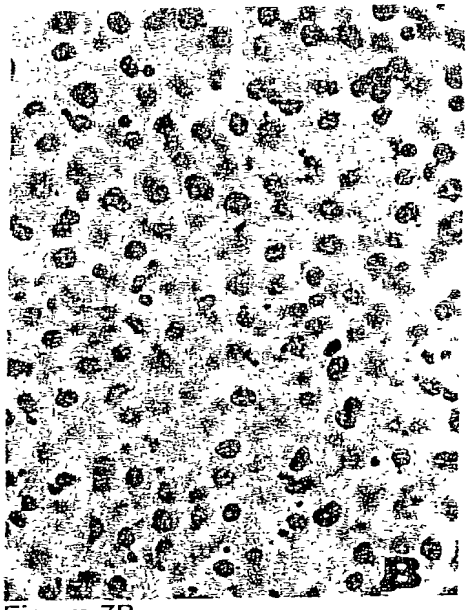
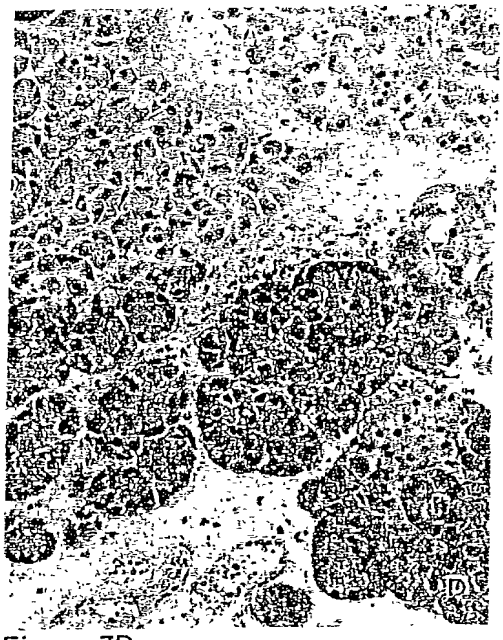


Figure 7B

Figure 7C



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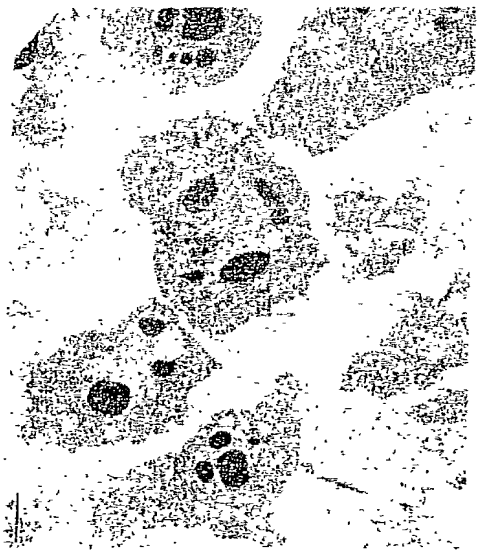

Figure 8

#4

DOCKET NO. 15317

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TREATMENT OF PANCREATIC DISEASE

the specification of which

(check one)

[ ] is attached hereto.

[X]was filed on 28 February 2002 as a United States Application No. or PCT International Application Serial No. PCT/AU00/01026
and was amended on _____.
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37 Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 (a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT international application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s):

Number	Country	Date of Filing Day/Month/Year	Priority Claimed Under 35 U.S.C. 119
PQ2536	Australia	30 August 1999	☒ YES ☐ NO
			☐ YES ☐ NO

I hereby claim the benefit under Title 35, United States Code, Section119(e) of any United States provisional application(s) listed below:

(Application Number) (Filing Date)

(Application Number) (Filing Date)

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35, United States Code, Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C.F.R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

PCT/AU00/01026	30 August 2000	Pending
Application Serial No.	Filing Date	Status
Application Serial No.	Filing Date	Status

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

(10)

Frank S. DiGiglio; Reg. No. 31,346
Paul J. Esatto, Jr.; Reg. No. 30,749
Mark J. Cohen; Reg. No. 32,211
Edward W. Grolz; Reg. No. 33,705
Steven Fischman; Reg. No. 34,594
Kenneth L. King; Reg. No. 24,223
Richard L. Catania; Reg. No. 32,608
Peter I. Bernstein; Reg. No. 43,497
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